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ORIGINAL ARTICLE

A Novel Turn on Fluorescence Sensor for Determination Enoxaparin, a Low Molecular Weight Heparin

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Abstract

A sensor system was designed for the detection of Enoxaparin (Enox), a low molecular weight heparin (LMWH) that was run over the fluorescence quenching mechanism of fluorescein (FL) dye. At nanomolar concentrations, FL probe was subjected to fluorescence quenching by Fe(III). Fluorescence quenching mechanism of FL by Fe(III) was examined using various analytical techniques such as UV-vis absorption, fluorescence, and Fourier transform Infrared spectroscopy techniques, as well as with scanning electron microscope. The results indicated that photoinduced electron transfer process occurred between FL and Fe and that FL was quenched both statically and dynamically. Thermodynamic parameters showed that the interactions between them were predominantly hydrophobic interactions. Enox caused FL to recover its lost fluorescence properties and an increase was observed in the intensity of the fluorescence. Enox was detected successfully with the turn on fluorescence sensor. The developed Enox biosensor exhibited linearity in the range of 0–1.1 μg/ml. For Enox detection, the limit of detection was measured as 255 ng/mL. Enox biosensor was presented as a practical, simple, and applicable sensor system with high sensitivity and good selectivity. Enox is a medication usually monitored indirectly over anticoagulation. This study was presented as an alternative method for monitoring Enox directly.

Highlights

- Fluorescence quenching of Fluorescein dye by Fe(III) was studied in detail.
- The presence of enoxaparin enhanced the fluorescence properties of the fluorescein dye.
- A sensitive, simple and effective sensor system for determination of Enoxaparin, a low molecular weight heparin was shaped in the aqueous media.
- It was presented as a new method for Enoxaparin to be followed directly.

Keywords Fluorescence sensor · Enoxaparin · Fluorescein dye, fluorescence quenching

Introduction

Presenting a recent alternative method in drug detection, the studies on monitoring of drugs with fluorescent factors and on drug detection using spectrophotometric and spectrofluometric methods have been increasing in numbers in the literature [1, 12, 16]. Majority of drug active ingredients do not have fluorescence properties. Drug can be monitored over a fluorescent probe by molecular interaction or it can also be monitored with the covalent bonds between the drug and the fluorescent probe [3, 8]. There are detection studies for the detection of active ingredients of anticoagulant drugs where spectrofluometric methods are employed. However, the number of such studies in the literature is very few [7, 9, 10, 28].

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Fluorescein (FL) bioscience is one of the most commonly used fluorescent probes. High quantum yield, high extinction coefficient, presence for a long time, and tendency to form conjugation with biomolecules makes this dye popular in studies in biologic environments [22]. FL is a very commonly used dye and there are studies in the literature on Fe(III) detection by synthesizing the derivatives of FL compound. A detection system was constituted for Fe(III) ion by synthesizing FL-based porous aromatic frame with post-modification [26]. FLN/OS-LDH and FLN/OS-LRH composites (FLN-fluorescein, 1-octane sulfonic acid sodium, LDH-Layered Double Hydroxide, and LRD-layered rare-earth hydroxides) were synthesized with ion exchange method and Fe(III) ion detection was made with the quenching in the presence of Fe(III) [38, 46]. An another study, Fluorescein-Desferrioxamine was synthesized for the detection of Fe(III) ions and was used for dosing [37]. Fe(III) complex was synthesized by treating certain surfactants with $FeCl_2$ and hexadeciltrimethyl ammonium chloride at the ratio of 1:1 and fluorescence quantum yield of FL was found to have increased [15]. From the conducted studies, it is known that FL derivatives and new constructs interact with Fe. However, quenching between FL and Fe(III) was not presented in the literature. In the present study, quenching of FL dye compound by Fe(III) and characterization of this quenching were presented. When Enox is added to the solution, fluorescence of the dye was recovered. This provided the optimum conditions for Enox detection.

Enox is a drug active ingredient in the low-molecular-weight heparin group that has higher bioavalability and longer half-life than unfractionated heparin (UFH). Enox, which is a low-molecular-weight heparin synthesized by alkaline beta division of benzyl ester of heparin, is an anticoagulant [18]. However, it is different from heparin regarding the action mechanisms. Enox is an effective alternative in the prophylaxis and treatment of thrombosis; ease of application compared to unfractionated heparin and lower tendency to haemorrhage complications are among its significant advantages [6].

Heparin detection studies were conducted using fluorescent indicator and constructs [20, 24, 25, 30, 33, 34, 40]. There are also studies on Heparin Sulfate, Heparin, and low-molecular-weight heparin (Enox) characterization and detection studies with chromatography techniques such as reverse-phase-high performance liquid chromatography [14]. However, the literature on the detection of Enox by spectrofluorometric methods is very limited. We have summarized several of these studies. Polycationic fluorescent probe Heparin Red used in the detection of UFHs (heparin), LMWHs (enoxaparin) and of non-anticoagulant heparins in the clinically relevant activity (in the range of 0.2–1.0 IU/ml) range using spectrophotometric and spectrofluometric method [44]. Fluorescent ruthenium complexes were designed and determined LMWH (0–10 IU/ml) by fluorescence assay on anti-xa activity in the buffer solutions [39].

Enox catalyzes the bonding of antithrombin III with both factor Xa and factor IIa. Activation of anti-factor Xa and anti-factor IIa results in anticoagulation. Anticoagulant activity of Enox can be monitored by measuring factor Xa inhibition (anti-factor Xa activity) [17]. This test is named as low-molecular-weight heparin test. Therapeutic range for anticoagulation is 0.5–1.0 IU/mL [19].

Studies performed in literature, Enox determination is done indirectly and over anticoagulation. It was presented in the current study as an alternative method for direct detection of Enox in aqueous medium at pH 7.4. A practical, non-toxic detection system with small amount of chemicals was constructed for Enox in aqueous mediums based on the quenching between FL and Fe(III). For Enox detection, fluorescence properties of the FL dye were observed and low limit of detection (LOD) was obtained.

Experimental

Materials

Fluorescein and Enoxaparin sodium was purchased from Sigma. Methanol was purchased from Merck. Trisma and NaCl using Trisma - HCl buffer (pH 7.4, containing 0.15 $molL^{-1}$ Tris and 0.05 $molL^{-1}$ NaCl) used to adjust the pH of the solutions was obtained from Sigma. Stock solutions of metal ions were prepared from their chloride or nitrate salts.

Apparatus and Measurements

Perkin-Elmer lambda 25 UV – vis spectrophotometer was used for absorbance measurements. Fluorescence measurements were recorded using a Shimadzu RF-5301PC spectrofluorophotometer equipped with a 1 cm path length quartz cuvette. The excitation and emission slits were set at 1.0 nm. Spectrofluorophotometer light source filters were used 1.5 and 3 for excitation and emission, respectively. Absorbance spectra were recorded between 400 and 550 nm. Fluorescence spectra were recorded between 468 and 650 nm using the excitation wavelength 465 nm.

The characterization of sensor system, Fluorescein-Fe-Enox samples were scanned using a FEI-Quanta FEG 450 Scanning electron microscope (SEM) and Agilent Cary 630 Fourier transformed infrared (FTIR) spectrophotometer.

Results and Discussion

Spectral Properties of FL with Metal Ions

FL dye compound has characteristically two absorption band maxima around 540 and 579 nm. It has a fluorescence maximum around 509 nm [41]. FL compound was treated with different metal ions at 7.4 pH tris HCl environment at 15 μM concentration; the corresponding absorption and fluorescence spectra are given in Fig. 1a and b, respectively. FL was not quenched except for Fe(III). Image under ultraviolet light is given in Fig. 1c.

Fluorescence Quenching Between FL and Fe(III)

Fluorescence quenching is a method used for the detection of neurotransmitters, virus antigens, and antibiotics [11, 27, 45]. There are two types of quenching mechanisms; namely collisional and static quenching. Collisional quenching is the result of diffusion of a quencher to fluorophore. Static quenching is dependent upon complex formation. Collisional quenching is expressed with the equation called Stern–Volmer equation [22].

$$I_0/I = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \quad (1)$$

In this expression K_{SV} is the Stern-Volmer quenching constant, and [Q] is the quencher concentration. The Stern-Volmer quenching constant K_{SV} shows the sensitivity of the fluorophore (FL) to a quencher Fe(III), k_q signified the FL bimolecular quenching rate constant, τ_0 signified the fluorescence lifetime of FL ($\tau_0 = 3.84\text{x}10^{-9}$ s) [41].

Figure 2a gives the quenching of FL dye compound by Fe(III). Fluorescence property decreased as Fe(III) was titrated into the solution. K_{SV} values were calculated using the Stern–Volmer equation and graphical data. Temperature and biomolecular quenching constant k_q values are important parameters for determining the quenching mechanism. Studies were repeated at different temperatures to determine the quenching mechanism. k_q was found and is presented in Table 1. K_{SV}

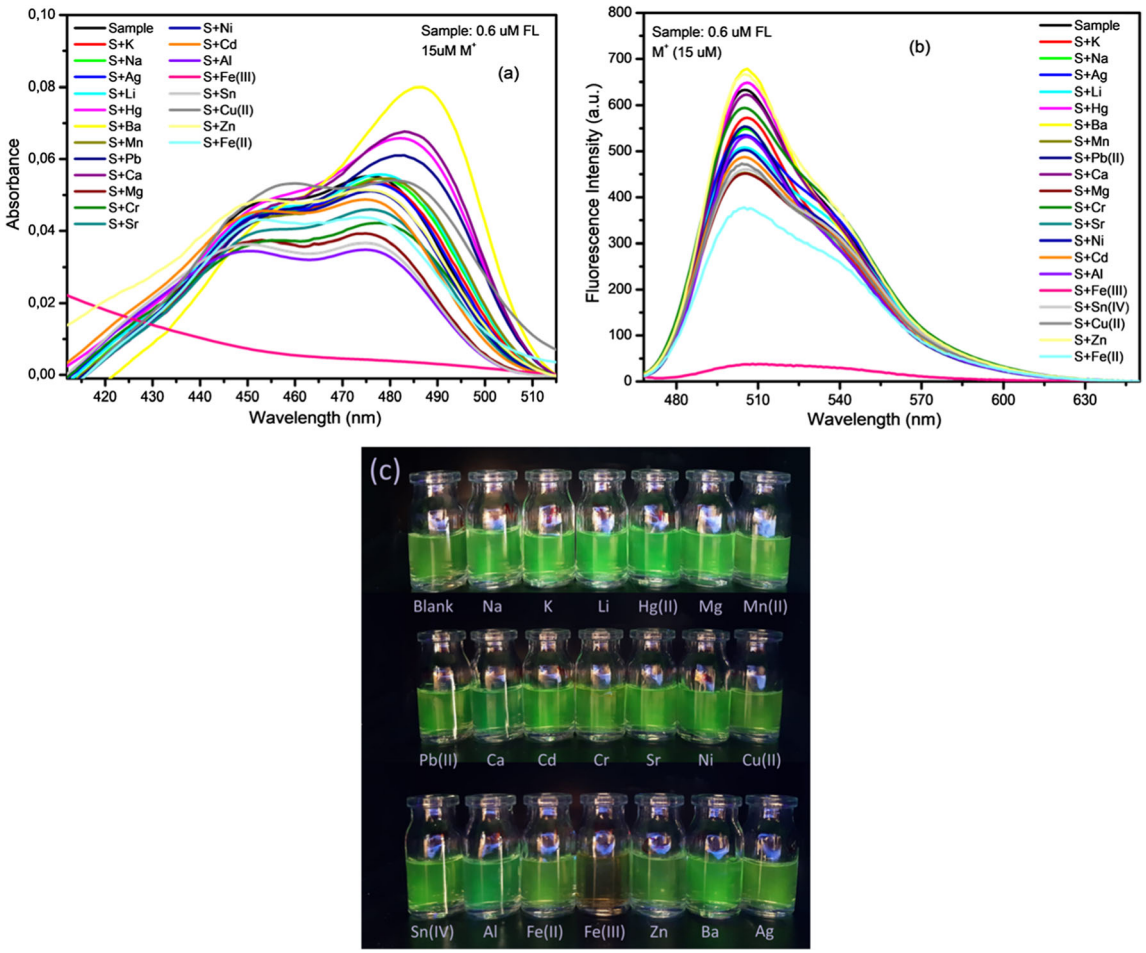

Fig. 1 (**a**) Absorbance and (**b**) fluorescence spectra of FL with different metal ions in aqueous solution, pH = 7.4; λ_{ex} = 465 nm, (**c**) Image of samples under UV light

Table 1 Stern-Volmer quenching constant (K_{SV}) and thermodynamic parameters of the FL-Fe (III) systems at different temperatures at pH 7.4

T (K)	$K_{SV} \times 10^7$ (M^{-1})	R^2	$k_q \times 10^8$ (M^{-1} s)	ΔH (K. J/mol)	ΔS (J/mol)	ΔG × 10^3 (K. J/mol)
283	1.4685	0.999	3.82	4980	154.93	−38.86
293	1.6300	0.999	4.24			−40.41
313	1.8376	0.999	4.77			−43.51

value increased with increasing temperature and a sharp linearity was obtained on the curves (Fig. 2b). K_q values were smaller than the maximal rate constant (2×10^{10} L/mol s) (Table 1). This situation implied that quenching occurred with dynamic quenching mechanism to a large extent [31]. However, the Stern–Volmer graphic was not linear at higher concentrations and had deviation (Fig. 2c). This situation implied that there was static quenching between FL and Fe(III). Consequently, FL was quenched with Fe(III) both dynamically and statically. With the Benesi-Hildebrand equation (Eq. 4), the binding constant and stoichiometry of Fe(III) were 222.0 ± 24.4 nM^{-1} and 1: 1, respectively (Fig. S1a). The detection limit for Fe(III) was determined with the calibration curve (Fig. S1b). For this purpose, the 3 s/k equation was used. The detection limit of Fe(III) was determined to be 400.0 ± 5.2 nM.

Driving forces between FL and Fe(III) were determined using van't-Hoff and Gibbs–Helmholtz equation. In the plot depicting log Ksv vs 1/T (Fig. 2d), ΔH and ΔS were calculated from the slope of linear and the intercept of the Y-axis, respectively.

$$\log K = -\frac{\Delta H}{2.303RT} + \frac{\Delta S}{2.303R} \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

ΔH; enthalpy change, ΔS; entropy change and ΔG; indicates free energy change. When ΔH > 0 ΔS > 0, the dominant interaction between molecules is hydrophobic interaction. ΔG

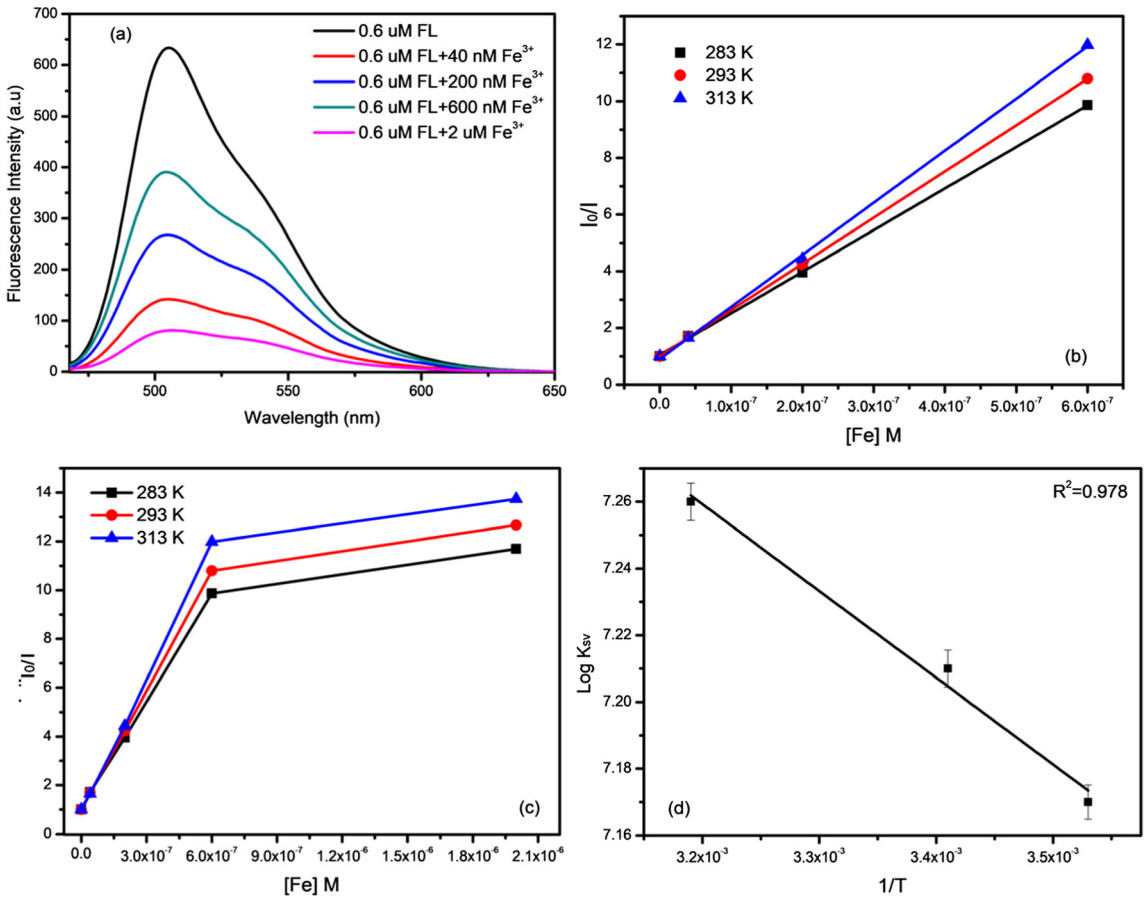

Fig. 2 (**a**) Fluorescence spectra of FL in various concentration of Fe (III) in aqueous solution, pH = 7.4; λ_{ex} = 465 nm (**b**) Stern-Volmer Plot in low concentrations of Fe (III), (**c**) Stern Volmer Plot including high concentrations of Fe(III), (**d**) Van't Hoff plot

value of the indicator is negative is that the event occurred spontaneously.

Detection of Enoxaparin

Detection and sensitivity with fluometric titration is a commonly used method. Fast response, practicality, and sensitivity of this method keep the interest in fluometric sensor studies alive. As seen in Fig. 3a, Enox was titrated into the medium containing 0.6 μM FL and 2 μM Fe(III) and fluorescence spectra were recorded. Lost fluorescence properties were recovered with increasing Enox concentration. The fluorescence intensity of the FL was recovered by 121%. A clear fluorescence expansion was observed in FL. It showed that bonding with Enox increased the rigidity of the dye and there was an interaction that inhibited the non-radiation relaxation means [2, 23]. It allowed the sensor to have a better signaling. Fluorescence intensity remained constant in concentration values higher than 1.1 μg/mL. The system gave a net response to the medication in the range of 0–1.1 μg/mL. Calculated using the Benesi–Hildebrand method, the binding constant and the stoichiometry of the resulting interaction were found to be 4.334 ± 0.044 ml μg^{-1} and 1:1, respectively (Fig. 3b). Benesi Hildebrand equation.

$$\frac{1}{(I-I_0)} = \frac{1}{K_B(I_{max}-I_0)[C]^n} + \frac{1}{(I_{max}-I_0)} \quad (4)$$

where I_0 and I; fluorescence intensity of FL in the absence and presence of Enox, respectively; $[C]^n$; concentration of Enox, I_{max}; fluorescence intensity in the complete interaction concentration of Enox, and K_b is the binding constant.

For Enox detection sensitivity of the system, both the LOD and the limit of quantification (LOQ) were calculated and evaluated using Miller's formulas [29]. For this purpose, the 3 s/k and 10s/k equations were used; in which s is the standard deviation of blank and k is the slope of the fit line in the fluorescence experiment. LOD and LOQ were calculated for the system as 255 and 728 ng/ml, respectively.

Mechanism of Fluorescence Extinction of FL

It has been shown recently that FL is a directly conjugated electron transmitter and receiver system. It is also known that fluorescence properties of FL derivatives are controllable with photoinduced electron transfer (PET) [37]. PET is a mechanism which is well-known for breaking the competition between bonding of ions and other types, between the de-excitation paths of fluorescence and electron transfer and whereby, fluorescence of fluorophore is quenched with electron transfer from the transmitter to the receiver [4]. According to molecular orbital theory, when FL bonds with a Fe(III) atom, electron transmitter HOMO energy level increases sufficiently to overcome the electron receiver HUMO energy level that allows the transfer of a single electron from the donor to the excited fluorophore. As a result, fluorophore loses its energy thermally instead of emitting fluorescence. However, when Fe(III) bonded with Enox, the energy level of FL returns to its free level and electron transfer is inhibited. But FL is more rigid because of molecular interactions. This

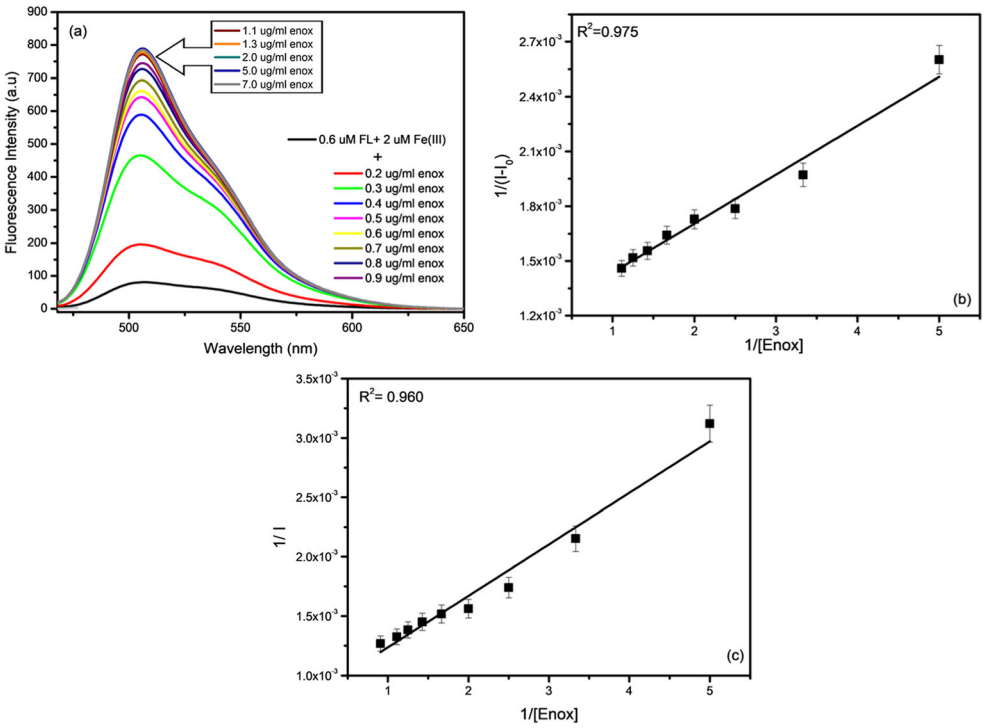

Fig. 3 (**a**) Fluorescence spectra of FL in the presence of different concentrations of Enox pH = 7.4; λ_{ex} = 465 nm. (**b**) Relative fluorescence intensity of Enox concentration, (**c**) Benesi Hildebrand plot

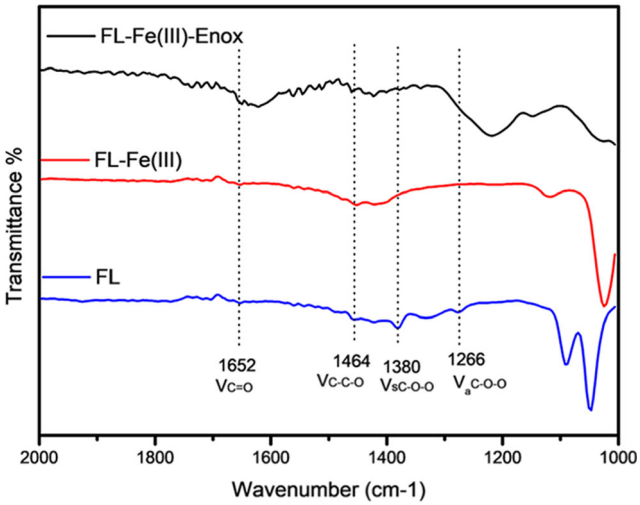

Fig. 4 FTIR spectrum of FL, FL-Fe(III) ve FL-Fe(III)-Enox solutions

situation causes a high fluorescence emission [37]. This situation is shown representatively in Fig. S2.

Characterization of Interaction Between FL, Fe^{3+}, Enox

Fluorescein exhibited a very broad and strong peak in the range of 3500–3000 cm^{-1}, due to the phenolic OH groups. Therefore the FTIR studies were focused on the frequency range from 1000 to 2000 cm^{-1} for underline changes in molecular symmetry [42]. The band at 1652 cm^{-1} was refer to the characteristic vibrations of the C=O group [38], while the bands at 1266 and 1380 cm^{-1} refer to asymmetric and symmetric COO^- stretching vibration of COOH groups. The band at 1464 cm^{-1} refer to C—C stretching vibration conjugated CO and CC stretch with symmetric COO^- stretch [43]. When Fe(III) ions and Enox were added to the solution respectively, the characteristic stretching vibrations COO^- (1266 and 1380 cm^{-1}) peak of FL disappeared (Fig. 4). This showed that Fe^{3+} ions and Enox molecules interacted with FL molecules side of COO^-. More clearly, the change of the COO^- characteristic bands showed that the Fe(III) has a molecular interaction with the carboxyl group of the FL compound. This showed that Fe(III) ions interacted electrostatically with FL. As a result of this interaction, the fluorescent emission of FL was quenched. The mechanism behind the FL-Fe (III) interaction may be the formation of a strong π electron interaction between the functional group of FL and the d-orbitals of the transition metal [13, 32, 35]. The proposed interaction mechanism with the effect of electrostatic interactions is given in Fig. S3.

Scanning electron microscope images taken from the solutions containing FL, FL-Fe(III), and FL-Fe(III)-Enox are shown in Fig. 5. Images are given on a scale of 500 nm. FL (Fig. 5a) was observed as a dispersed stack of small particles in different sizes. Dispersed FL particles exhibit a structuring of large size together with Fe(III) (Fig. 5b). Enox, FL, and Fe formed a large structuring (Fig. 5c). Mainly free FL particles were observed in the environment.

pH and Temperature Studies of FL-Fe-Enox System

FL is a dye with pH sensitivity. It is found in different pH solutions in cation, neutral, anion, and dianion forms. This situation makes its absorption and fluorescence properties dependent on pH. Different pH-dependent structural forms of FL are shown in Fig. S4 [5]. In mediums of around pH 2, i.e., in strongly acidic mediums, the dye is in cationic or neutral forms; in weak acidic or neutral mediums, it is in neutral, anionic, or dianionic forms [36]. However, in neutral mediums, monoanionic form is dominant by 85% over the other forms. The dye is entirely in dianionic form in alkaline mediums of pH 9.1 and above [21]. These structural changes make it important to determine the response of the system constituted for the detection of Enox to pH. The dye is predominantly in cationic form in acidic mediums. This situation adversely affects the interaction of Fe(III) and rate of free dye increased [2]. It is observed that the system exhibits a higher fluorescence response (Fig. 6). In alkaline mediums the response is relatively lower compared to neutral and acidic mediums (Fig. 6). Also, the response of FL and FL- Fe(III) systems to pH is given in Fig.S5.

The sensor system gave a relative clearer response at 283 K temperature. However, in the temperature range of 293–313 K, which is suitable from biological point of view, the system remained stable against temperature changes.

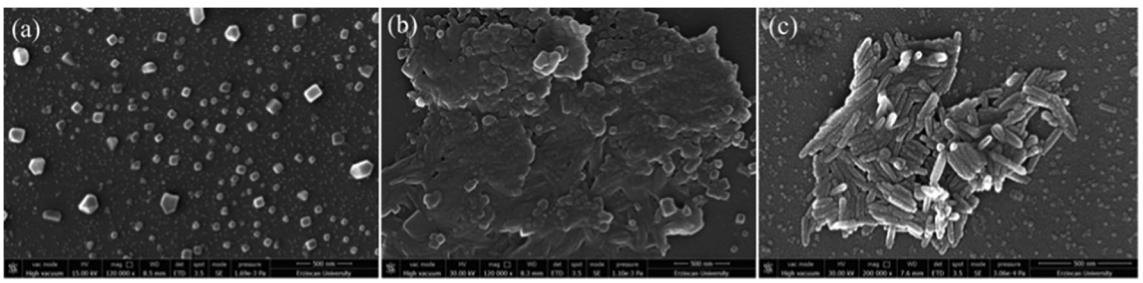

Fig. 5 SEM images of Fluorescein (**a**), Fluorescin-Fe (III) (**b**) Fluorescein-Fe (III)-Enoxaparin (**c**)

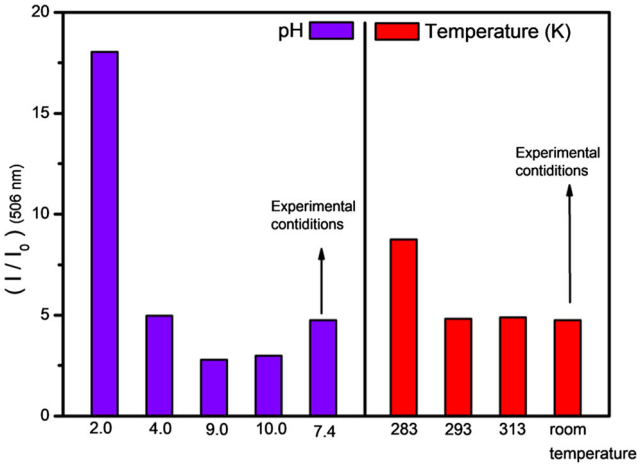

Fig. 6 Relative fluorescence intensity with different pH and temperatures

Selectivity and Interference Studies of FL-Fe-Enox System

Studies were conducted with aqueous solutions a few of the main substances that can be in the blood such as serum albumin, uric acid, urea, ascorbic acid, glucose to determine the selectivity and operability of FL–Fe(III)–Enox sensor system. It has been established that the system is selective and operable; the response of the system to other compounds are given in Fig. 7a and b with the relative fluorescence intensities.

The effect of metal ions on the sensor system was studied. It was determined that the metal ions did not show a noticeable effect on this enox detection system (Fig.7c). In addition, the effect of other metal ions on the quenching of FL was studied and given in Fig. S6.

Recovery Studies

Recovery tests were conducted to verify the accuracy of the method proposed for the detection of Enox. For this purpose, the medicine Oksapar 40 mg enoxaparin sodium (equivalent to 4000 anti-Xa IU) in the form of injection, whose active ingredient is enoxaparin sodium, was added to the samples and the fluorescence spectra were taken under the same experimental conditions. The recovery values given in Table 2 are satisfactory.

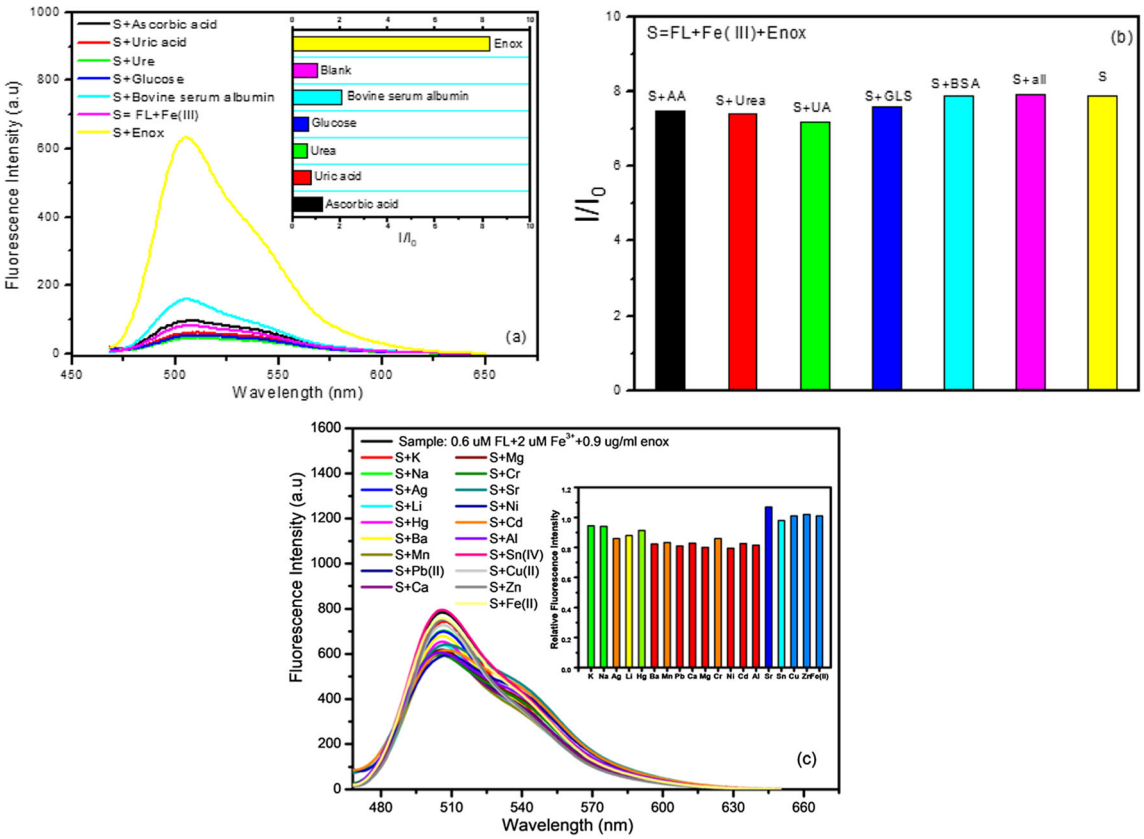

Fig. 7 (**a**) The fluorescence spectra of the FL–Fe system to Enox and other biomolecules (**b**) Relative fluorescence response of FL–Fe-Enox sensor system with other biomolecules, (**c**) The fluorescence spectra and relative fluorescence response of the FL–Fe-Enox sensor system to metal ions

Table 2 Recovery values of Enox solutions

Enox μg/ml		
Calculated	Found	Recovery
0.2	0.22	110.00[a]±1.3
0.5	0.52	104.00[a] ± 1.1
0.8	0.85	106.25[a] ± 0.6

[a] The mean of three measurements

Conclusion

In conclusion, FL has shown detection properties for Fe(III) compared to other metal ions and lost its existing fluorescence property. A quenching occurred between FL and Fe(III), where static and dynamic quenching occurred together. Thermodynamic parameters were calculated and it was found that hydrophobic interactions were dominant. In the presence of Enox, FL recovered its fluorescence properties. This "turn on" type fluorescence sensor with high selectivity and sensitivity was constructed and was used successfully for the quantitative detection of Enox with detection limits of 255 ng/ml.

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Compliance with Ethical Standards

Declaration of Competing Interest The authors declare that they have no conflict of interest.

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